Neuron, Vol. 16, 665–673, March, 1996, Copyright ©1996 by Cell Press

GDNF Induces a Dystonia-like State in Neonatal Rats and Stimulates Dopamine and Serotonin Synthesis

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Summary

To test whether glial cell line–derived neurotrophic factor (GDNF) regulates the development of nigral dopaminergic neurons in vivo, neonatal rats received bilateral injections of GDNF into the striatum. Injections at postnatal day 2 induced a unique transient behavioral pattern characterized by forelimb hyperflexure, clawed toes of all limbs, and a kinked tail. Parallel to the behavioral changes, the levels of striatal and ventral mesencephalic dopamine and serotonin were increased from 60% to 100% with a proportional increase of principal metabolite levels. GDNF increased tyrosine hydroxylase activity in the ventral mesencephalon, but did not affect striatal activity of choline acetyltransferase and GABA uptake. GDNF failed to induce sprouting of dopaminergic neurites. Our findings suggest that during development striatal GDNF regulates the capacity of dopaminergic and of serotonergic neurons for neurotransmitter production and release.

Introduction

Dopaminergic neurons in the ventral mesencephalon play a crucial role in the regulation of motor function, and there is evidence for their involvement in cognitive processes. The degeneration of dopaminergic neurons represents the defining feature of Parkinson's disease. The development of this neuronal population has been described in detail in the rat. Initial innervation of the principal target areas (striatum, nucleus accumbens, olfactory tubercle, and cortex) occurs prenatally. The projection matures during the first 2 weeks postnatally and exhibits a gradual increase in terminal density in the target areas toward adult levels (Specht et al., 1981a, 1981b; Voorn et al., 1988; Broaddus and Bennett, 1990; Arbogast and Voogt, 1991).

A number of growth factors have been found to increase survival or differentiation (or both) of cultured embryonic dopaminergic neurons (Beck, 1994; Poulsen et al., 1994; Krieglstein and Unsicker, 1994); however, glial cell line–derived neurotrophic factor (GDNF) has been the only factor significantly protecting dopaminergic neurons from lesion-induced degeneration in vivo (Hoffer et al., 1994; Beck et al., 1995; Kearns and Gash, 1995; Sauer et al., 1995; Tomac et al., 1995a). In the CNS, the pattern of GDNF mRNA expression strongly suggests a role for this factor in the development of the nigrostriatal projection. During dopaminergic axon outgrowth and initial target innervation in developing rats (embryonic day 15.5 [E15.5]) GDNF mRNA is expressed in the ventral mesencephalon in close proximity to the dopaminergic neurons. GDNF mRNA transcripts are also expressed outside the CNS, suggesting possible functions during oraganogenesis, innervation, or both (Suter-Crazzolara and Unsicker, 1995). During postnatal development, GDNF mRNA is undetectable in the mesencephalon, but is transiently expressed in the striatum, the innervation target of nigral dopaminergic neurons (Schaar et al., 1993; Stroemberg et al., 1993; Poulsen et al., 1994). In adult rat brain, GDNF mRNA is undetectable, but its expression can be induced by limbic motor status epilepticus in striatum, hippocampus, and cortex (Schmidt-Kastner et al., 1994; Ho et al., 1995). A recent report demonstrated retrograde transport of iodinated GDNF to nigral dopaminergic neurons following injection into the striatum of adult rats (Tomac et al., 1995b).

The GDNF mRNA expression pattern suggests that striatal GDNF regulates the maturation of dopaminergic terminals in this target area. To test this hypothesis directly, we increased striatal GDNF levels during postnatal development by injecting rat recombinant GDNF into this brain area. The initial observations revealed striking behavioral consequences that were then found to be associated with the augmentation of biochemical parameters of dopaminergic and serotonergic neuron function.

Results

Single bilateral injections of 10 μg of GDNF (5 μg each side) into the striatum of postnatal day 2 (P2) rats produced a striking and unique pattern of behavioral changes (Figure 1). Most prominent was the smaller size of GDNF injected rats and the appearance of hyperflexed forelimbs that was maintained during all behavioral activities (Figures 1A, 1E, and 1F). When lifted up, GDNF injected pups did not extend their forelimbs toward a surface as normal animals do (Figure 1B). A second obvious GDNF-induced behavioral difference related to the appearance of the tails of the rats. Tails of pups injected with a control protein showed a smooth curvature of the tail as did uninjected rats (Figures 1A and 1C). In contrast, GDNF injected rats showed multiple kinks in their tails, reflecting increased muscle tension (Figures 1A and 1D). Third, GDNF injected animals kept their toes in a clawed position (Figures 1A, 1D, and 1F). This phenomenon was observed both at forelimbs and hindlimbs. There was no obvious difference in locomotor activity between GDNF injected and control rats; however, control rats were more successful in gaining access to suckle. There were no obvious behavioral differences between animals receiving control protein

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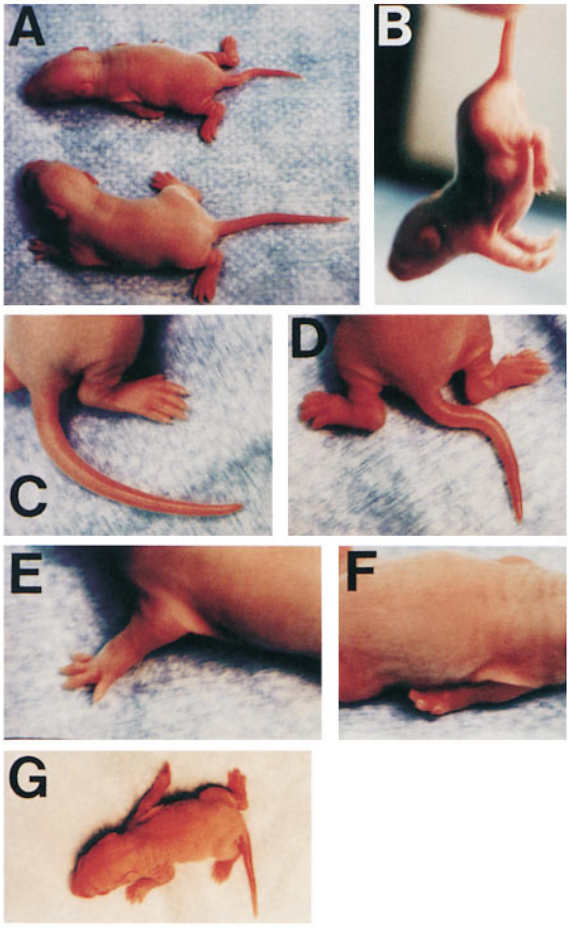

Figure 1. Increased Muscle Tonus Following Intrastriatal Injection of GDNF

Shown are rat pups at P8 that had received bilateral intrastriatal injections of GDNF at P2.
(A) Comparison to a cytochrome C injected littermate (bottom). Note reduced body size and hyperflexed forelimbs in GDNF injected pup.
(B) When lifted up, GDNF injected pups do not extend their forelimbs.
(C–G) Tails of cytochrome C injected pups are smoothly curved (C), while GDNF injected rats show multiple kinks and toes are kept in a clawed position (D). Close up of forelimbs show normal positioning (E) and hyperflexion after GDNF injection (F); forelimbs were kept in this position during all activities. Unilateral striatal GDNF injections induce contralateral forelimb hyperflexion (G).

injections and uninjected controls. The behavioral changes became evident 3–4 days after the injections (P5–P6) and were most evident in the period between 6–12 days after the injections (P8–P14). They gradually declined in intensity thereafter and were no longer present 26 days after the GDNF injections (P28). Rats injected with 5 μg of GDNF could not be behaviorally distinguished from rats injected with 10 μg. While administration of 0.05 μg of GDNF did not produce behavioral changes, rats injected with 0.5 μg displayed slight paw clawing and tail kinking, but no forelimb hyperflexion (data not shown). Bilateral injection of a total of 10 μg of GDNF above the substantia nigra of P2 rats produced identical transient behavioral changes (data not shown). In addition, one group of neonatal animals (P2) was given unilateral striatal injections of 5 μg GDNF. Different from rats with bilateral injections, these unilaterally injected rats developed hyperflexure limited to the forelimb and, to a lesser degree, also the hindlimb contralateral to the injected side without an effect on the ipsilateral side (Figure 1G). In contrast with neonatal rats, adult rats receiving single GDNF injections (10 μg) into the striatum or above the substantia nigra were unaffected in posture and behavior. Also the levels of dopamine, serotonin, and their respective metabolites were unaffected (Table 1).

GDNF-treated neonatal rats showed a slower rate of weight increase compared with cytochrome C injected and uninjected controls. There were significant differences in weight between GDNF-treated and control groups at 6 and 12 days (P8 and P14) after the intrastriatal injections (Figure 2). In parallel to the behavioral changes, the weight differences were no longer evident 26 days after the injections (P28).

To test for the hypothesis that the behavioral changes reflect alterations in the function of nigrostriatal neurons, we measured several biochemical and morphological dopaminergic parameters. Levels of striatal and ventral mesencephalic dopamine (DA) at P8 were increased by 60% and 100%, respectively, in GDNF-treated animals as compared with cytochrome C injected controls (Table 1). At P14, the increases were reduced to 25% and 37%, respectively, and at P28, no significant difference could be detected. Levels of the principal DA metabolites, DOPAC and HVA, were increased proportionally to those of dopamine itself. Striatal and ventral mesencephalic levels of serotonin (5-HT) and its metabolite hydroxyindolaceticacid (HIAA) were increased by GDNF in parallel to the increases seen in DA and its metabolites (Table 1). Compared with cytochrome C injected rats, at P8 the striatal and ventral mesencephalic levels of 5-HT were increased by 85% and 95%, respectively. At P14 in GDNF injected rats, striatal 5-HT levels were not significantly different from those in cytochrome C injected controls, but there was a 28% increase in the mesencephalon. At P28, no significant differences could be detected. Bilateral GDNF injection into the midbrain above the substantia nigra produced increases of DA, 5-HT, and metabolites in P8 striatum and ventral mesencephalon similar to those seen after striatal administration (Table 1).

Figure 3 shows the results of a dose-response analysis of the GDNF effect on tissue DA (Figure 3A) and 5-HT levels (Figure 3B) at P8. Striatal injection of 0.05 μg of GDNF did not affect DA levels, while 0.5 μg increased DA by 25% and 29% above levels measured in striatum and ventral mesencephalon, respectively, of animals injected with control protein. Following 5 μg of GDNF, striatal DA was increased by 57% and mesencephalic dopamine by 95%. DOPAC and HVA levels were increased proportionally (data not shown). 5-HT levels were increased by GDNF in a similar fashion (Figure 3B): 0.05 μg of GDNF produced no significant changes, 0.5 μg increased striatal and mesencephalic 5-HT by 37% and 45%, respectively, while 5 μg of GDNF produced a maximal increase of 55% and 88%, respectively. HIAA levels were increased proportionally (data not shown).

To test whether the increased DA levels reflect the

Table 1. Tissue Levels of DA, 5-HT, and Metabolites Following GDNF Injections into the Striatum or above Substantia Nigra in Neonatal (5 μg, Bilateral) and in Adult Rats (10 μg, Unilateral)

Test	DA	DOPAC	HVA	5-HT	HIAA
P8,striatal injection					
Striatum					
Control (10)	40.11 ± 4.34	6.22 ± 0.57	6.24 ± 0.80	2.50 ± 0.26	4.51 ± 0.32
Cytochrome C (10)	31.50 ± 7.20	5.80 ± 0.85	6.55 ± 0.70	2.55 ± 0.23	4.19 ± 0.58
GDNF (10)	52.98 ± 4.16[b]	8.65 ± 0.75[b]	9.87 ± 0.71[b]	4.72 ± 0.39[b]	7.82 ± 0.70[b]
Mesencephalon					
Control	11.44 ± 1.03	6.52 ± 0.65	6.26 ± 0.85	9.51 ± 1.07	15.97 ± 1.69
Cytochrome C	8.43 ± 1.05	4.41 ± 0.41	4.20 ± 0.47	8.47 ± 0.61	13.20 ± 1.42
GDNF	16.89 ± 1.31[b]	7.29 ± 0.69[b]	7.87 ± 0.60[b]	16.55 ± 1.70[b]	24.01 ± 2.04[b]
P14,striatal injection					
Striatum					
Control (6)	61.71 ± 4.66	13.47 ± 0.59	13.80 ± 0.88	2.92 ± 0.32	10.14 ± 0.75
Cytochrome C (6)	57.46 ± 6.86	12.19 ± 1.30	14.94 ± 1.20	3.55 ± 0.34	9.41 ± 0.80
GDNF (6)	72.05 ± 5.01[b]	14.23 ± 1.23	14.05 ± 1.64	3.80 ± 0.30	10.31 ± 1.02
Mesencephalon					
Control	13.28 ± 0.88	6.22 ± 0.70	7.82 ± 0.68	16.39 ± 1.36	23.61 ± 1.84
Cytochrome C	14.24 ± 2.63	6.22 ± 0.88	6.88 ± 0.98	14.10 ± 1.03	20.16 ± 1.45
GDNF	19.50 ± 1.39[b]	9.86 ± 1.75[b]	9.25 ± 1.12[a]	18.04 ± 1.69[a]	24.59 ± 2.31[a]
P28, striatal injection					
Striatum					
Control (6)	72.45 ± 4.64	16.29 ± 2.89	14.10 ± 0.40	5.65 ± 0.61	13.16 ± 1.73
Cytochrome C (6)	72.71 ± 5.60	16.46 ± 2.43	13.16 ± 1.60	5.98 ± 0.51	11.66 ± 1.07
GDNF (6)	70.12 ± 7.54	16.81 ± 2.44	13.11 ± 1.60	7.00 ± 0.56	12.74 ± 1.26
Mesencephalon					
Control	9.49 ± 1.02	4.33 ± 0.21	4.39 ± 0.40	15.07 ± 1.46	33.76 ± 3.12
Cytochrome C	8.14 ± 1.01	4.10 ± 0.31	4.70 ± 0.23	13.29 ± 1.19	29.35 ± 2.37
GDNF	10.89 ± 1.09	4.19 ± 0.35	4.46 ± 0.57	15.40 ± 1.62	34.38 ± 3.34
P8, supranigral injection					
Striatum					
Cytochrome C (5)	40.80 ± 3.98	5.65 ± 0.41	6.01 ± 0.52	3.06 ± 0.30	7.64 ± 0.70
GDNF (5)	69.93 ± 5.92[b]	8.84 ± 0.66[b]	8.99 ± 0.88[b]	4.43 ± 0.32[b]	10.46 ± 0.98[b]
Mesencephalon					
Cytochrome C	9.04 ± 1.02	5.30 ± 0.36	4.74 ± 0.35	10.14 ± 1.18	18.70 ± 2.03
GDNF	16.94 ± 1.53[b]	8.46 ± 0.48[b]	7.66 ± 0.71[b]	15.19 ± 0.96[b]	25.67 ± 2.37[b]
Adult, 21 days after unilateral striatal injection					
Striatum					
Cytochrome C (4)					
Control side	85.33 ± 7.53	15.04 ± 2.01	8.94 ± 0.55	18.98 ± 1.44	18.12 ± 1.66
Injected side	86.80 ± 8.88	14.54 ± 1.22	8.65 ± 0.80	19.24 ± 1.86	18.81 ± 1.98
GDNF (4)					
Control side	84.45 ± 5.62	16.00 ± 1.22	8.66 ± 0.72	18.36 ± 1.28	18.88 ± 1.94
Injected side	86.68 ± 7.55	14.81 ± 1.31	8.50 ± 0.52	18.33 ± 1.86	18.01 ± 1.22
Mesencephalon					
Cytochrome C (4)					
Control side	9.42 ± 1.00	4.12 ± 0.33	4.09 ± 0.39	38.89 ± 3.91	42.03 ± 4.10
Injected side	10.02 ± 0.96	4.20 ± 0.40	4.23 ± 0.32	39.92 ± 3.12	45.41 ± 4.33
GDNF (4)					
Control side	10.22 ± 0.99	4.45 ± 0.40	4.22 ± 0.49	41.74 ± 3.99	43.11 ± 4.58
Injected side	9.89 ± 0.88	4.34 ± 0.41	4.24 ± 0.28	42.99 ± 3.84	42.98 ± 3.97
Adult, 21 days after unilateral nigral injection					
Striatum					
Cytochrome C (4)					
Control side	82.53 ± 3.62	14.07 ± 1.44	8.44 ± 1.19	20.06 ± 1.68	18.89 ± 1.69
Injected side	78.62 ± 3.88	13.92 ± 0.24	9.10 ± 0.57	19.70 ± 1.31	18.19 ± 1.64
GDNF (4)					
Control side	82.99 ± 7.11	14.04 ± 0.35	8.48 ± 0.47	21.15 ± 1.81	18.19 ± 1.64
Injected side	79.92 ± 4.47	12.61 ± 0.81	8.57 ± 0.50	20.06 ± 1.50	18.99 ± 1.65
Mesencephalon					
Cytochrome C (4)					
Control side	10.24 ± 0.90	4.42 ± 0.39	4.10 ± 0.39	41.03 ± 4.04	41.72 ± 4.00
Injected side	10.89 ± 1.02	4.68 ± 0.41	4.22 ± 0.41	39.21 ± 3.00	40.35 ± 3.10
GDNF (4)					
Control side	10.66 ± 1.14	4.55 ± 0.44	4.31 ± 0.36	39.98 ± 1.10	42.09 ± 2.39
Injected side	11.44 ± 1.10	4.68 ± 0.43	4.28 ± 0.40	42.41 ± 4.27	45.42 ± 3.28

All values are given as mean ± SEM (in ng/mg of protein). Number of animals per group are indicated in parentheses. Footnotes indicate statistically significant difference compared with corresponding cytochrome C injected group ([a]$p < 0.01$; [b]$p < 0.001$; Student's t test).

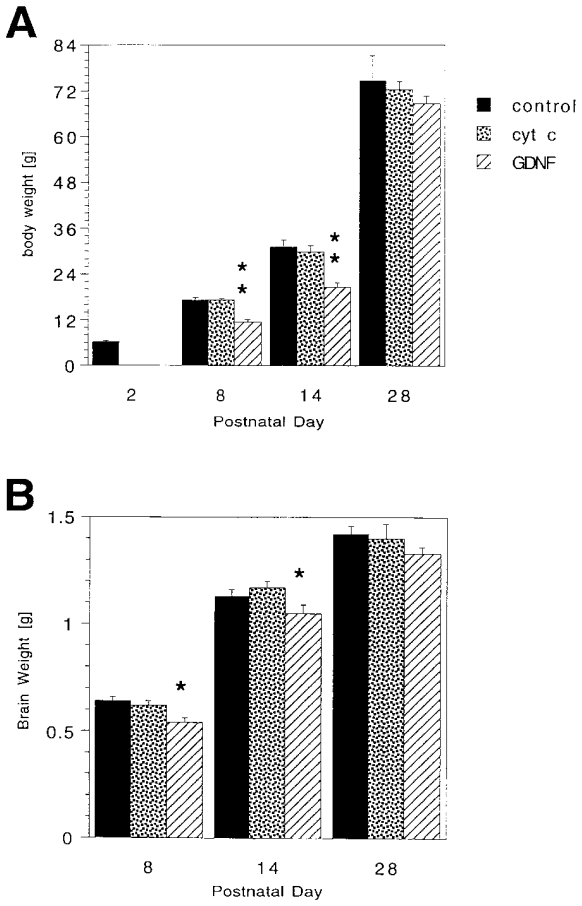

Figure 2. Postnatal Increase in Body and Brain Weights

Data for body (A) or brain (B) weights from control rats (closed bars) and from rats following injection of GDNF (dotted bars) or cytochrome C (hatched bars). Asterisks indicate statistically significant differences from corresponding control and cytochrome C groups (one asterisk, $p < 0.05$; two asterisks, $p < 0.01$; Student's t test).

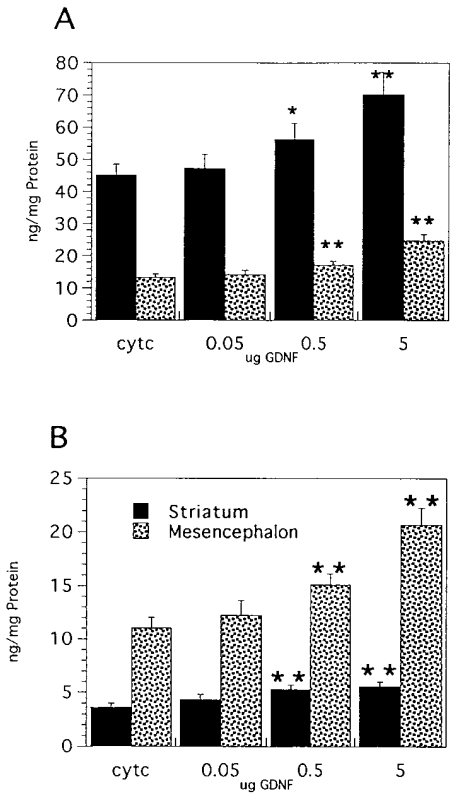

Figure 3. GDNF Dose-Response Analysis

DA (A) and 5-HT (B) levels in P8 rats after bilateral striatal injection of GDNF. Equal volumes of GDNF dilutions were injected at P2, and DA/5-HT levels were determined in P8 striata and ventral mesencephali as described in Experimental Procedures. Asterisks indicate statistically significant differences from corresponding control and cytochrome C groups (one asterisk, $p < 0.05$; two asterisks, $p < 0.01$; Student's t test). P8 rats injected with 0.05 μg GDNF did not show any behavioral changes, those injected with 0.5 μg displayed slight paw clawing and tail kinking, but no forelimb hyperflexion. The group injected with 5 μg GDNF showed the full dystonia-like pattern seen after 10 μg GDNF.

induction of synthesizing enzyme in dopaminergic neurites, we measured tyrosine hydroxylase activity in striatum and ventral mesencephalon of GDNF injected animals and visualized this enzyme with immunohistochemical methods. An increase in tyrosine hydroxylase (TH) activity (48%) was found in the mesencephalon but not striatum (Table 2). The activity of choline acetyltransferase (ChAT, a marker enzyme for striatal cholinergic interneurons) in striatal tissue and γ-aminobutyric acid (GABA) uptake into striatal synaptosomes were not affected by GDNF administration (Table 3).

No differences between GDNF-treated and control animals were observed in TH staining pattern and intensity, failing to support the hypothesis that GDNF stimulates sprouting of dopaminergic neurites (Figure 4). Aberrant TH-immunopositive fibers were not found in any brain area, excluding the possibility of significant sprouting of dopaminergic neurites into nontarget areas. The numbers and average cell body sizes of mesencephalic TH-positive neurons were not affected by GDNF treatment (Table 4).

[^{3}H]Mazindol-binding site density served as an additional parameter to test for changes in dopaminergic terminal density (Javitch et al., 1984). This parameter was moderately increased by GDNF at P8 (40%, Table 5), but was back to control levels by P14, when the behavioral changes still were maximal. The absence of strong changes of mazindol-binding site density and density of TH-positive fibers in the striatum does not support the view that the more robust increases in dopamine levels reflect sprouting of nigrostriatal terminals.

Table 2. Tyrosine Hydroxylase Activity after Intrastraital GDNF Injections (5 μg, Bilateral) into Neonatal Rats

Tissue	Control (9)	Cytochrome C (7)	GDNF (7)
Striatum	79.17 ± 6.88	75.35 ± 7.98	82.79 ± 7.56
Mesencephalon	80.69 ± 8.14	77.46 ± 5.62	115.09 ± 6.85[a]

Values are means ± SEM (in pmol of DOPA/mg of protein/min) from tissues at P8; numbers of rats per group are indicated in parentheses.

[a] Statistically significant difference compared with corresponding cytochrome C injected group ($p < 0.001$; Student's t test).

Table 3. Striatal ChAT Activity and GABA Uptake after Intrastriatal GDNF Injections (5 μg, Bilateral) into Neonatal Rats

Test	Control	Cytochrome C (5)	GDNF (8)
ChAT activity	9.60 ± 0.81 (6)	8.76 ± 0.75 (5)	9.51 ± 0.59 (8)
[^{3}H]GABA uptake	251.2 ± 12.1 (4)	239.2 ± 18.8 (5)	244.5 ± 20.3 (5)

Values are means ± SEM (in pmol of ACh or [^{3}H]GABA/mg of protein/min) from tissues at P8; numbers of rats per group are indicated in parentheses.

Also the the distribution and density of 5-HT immunostaining in the striataum and ventral mesencephalon was unaffected by GDNF (data not shown).

Discussion

The unique behavioral pattern induced by GDNF injections into the striatum of neonatal rats represents the most significant finding of the present study. GDNF induced transient hyperflexure of forelimbs and the appearance of clawed toes and kinked tails. The behavioral alterations were associated with increases in DA levels but not markers of dopaminergic fiber density, suggesting that they were caused by increased DA metabolism rather than sprouting of dopaminergic terminals. The transient increase in DA levels was accompanied by a parallel elevation of 5-HT tissue levels, but no altered distribution of 5-HT immunostaining.

The combination of behavioral changes induced by intrastriatal GDNF has not been described before. The pattern is reminiscent of the clinical condition dystonia, which is characterized by muscular hypertonus and rigidity (Adams and Victor 1993). Dystonia is a CNS dysfunction rather than a disorder of muscles. There are hereditary and idiopathic forms of dystonia, and the disorder can be generalized or limited to specific muscle groups. The etiologies of the different clinical forms of dystonia are largely unclear, but a possible involvement of dopaminergic mechanisms has been proposed based on the fact that patients suffering from generalized dystonias usually get some symptomatic relief from treatment with centrally acting dopamine antagonists, muscarinic agonists, or in more progressed cases, with L-DOPA (Adams and Victor, 1993).

Our biochemical analysis showed effects of GDNF on dopaminergic and, in addition, also on serotonergic neurons. Both transmitter systems are interacting extensively as suggested by the reciprocal anatomical projections between dopaminergic neurons in substantia nigra/VTA and serotonergic neurons in the dorsal raphe

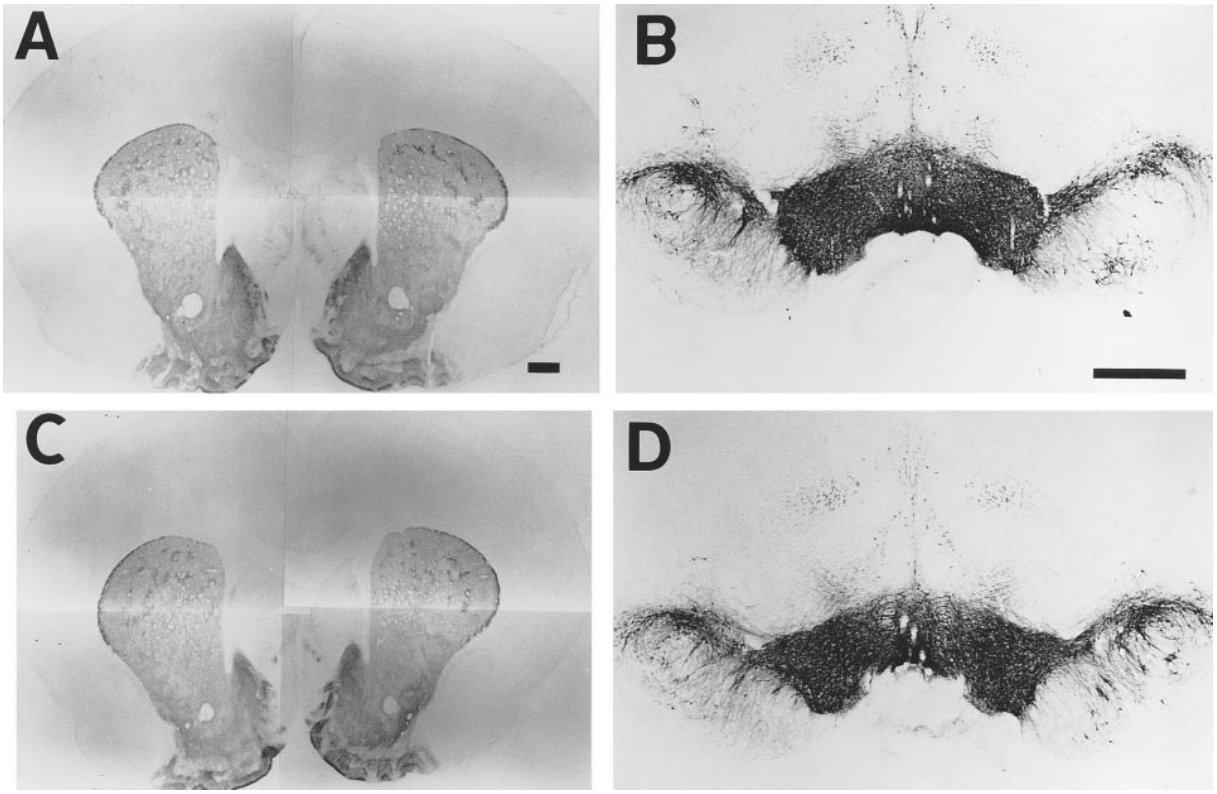

Figure 4. TH Immunostaining

Coronal brain sections at corresponding levels of striatum (A and C) and mesencephalon (B and D) of P8 rats following injection of cytochrome C (A and B) or GDNF (C and D). Pattern and intensity of TH staining is not significantly different; there was no aberrant TH staining throughout the brain.

Table 4. Numbers and Cell Body Sizes of Nigral TH-Positive Neurons after Neonatal GDNF Administration

Group	Number of Nigral TH-Positive Neurons	Cell Body Size (μm^2)
Control (5)	12,944 ± 843	80.9 ± 3.2
Cytochrome C (5)	12,252 ± 710	80.2 ± 4.5
GDNF (6)	12,452 ± 907	83.1 ± 3.9

Values are means ± SEM of cell counts from both sides with each sixth section used for analysis and multiplication of the result by six. Average cell sizes were determined from ten randomly selected nigral TH-positive neurons per rat and per three corresponding levels (30 cells per rat in total). Number of rats per group is indicated in parentheses.

nucleus (DRN; Dray et al., 1976; Pasquier et al, 1977; Azmitia and Segal, 1978; Herve et al., 1987; Kalen et al., 1988; Nedergaard et al., 1988; Van Bockstaele et al., 1993). Pharmacological interactions between both transmitter systems have been studied extensively using DA and 5-HT agonists and antagonists and intracranial microdialysis probes in freely moving rats, but the results were rather controversial (Benloucif and Galloway, 1991; Huang and Nichols, 1993; Ferre et al., 1994; Yadid et al., 1994; Dewey et al., 1995). Iontophoretic administration of 5-HT to the SN reduced electrical activity of nigral dopaminergic neurons, and electrical stimulation of the DRN has been reported to both activate and inhibit SN neurons (Dray et al., 1978). Recently, using positron emission tomography in baboons, Dewey et al. (1995) reported data consistent with an inhibitory action of 5-HT on striatal DA release. However, the net effect of 5-HT on dopaminergic neuron activity and DA release might be dependent on the functional status of dopaminergic neurons. In turn, there is also evidence for modulatory actions of DA on serotonergic neurons in the DRN: increased DA levels in the DRN inhibit the activity of serotonergic neurons projecting to the striatum via local 5-HT release and autoreceptor stimulation within the DRN. However, striatal and systemic administration of dopaminergic agonists does not affect striatal 5-HT release (Ferre et al., 1994). These data, together with the important finding that GDNF does not affect serotonergic neurons cocultured with dopaminergic neurons (Lin et al., 1993; Poulsen et al, 1994), suggest that the primary action of GDNF in our study is on developing dopaminergic neurons. The concommittant increase of 5-HT levels is most likely secondary and reflects an adaptive upregulation of serotonergic transmitter synthesis inhibiting dopaminergic neuron activity and DA release. However, based on our analysis, we cannot completely exclude a potential direct action of GDNF also on serotonergic neurons.

In contrast with the findings in neonatal rats, in adult rats with single unilateral injections of 10 μg of GDNF into the striatum or above the substantia nigra, we did not observe a significant weight loss (data not shown), no change in nigral and striatal TH immunoreactivity, no sprouting of TH-positive fibers toward the injection site, and no change in DA or 5-HT synthesis or turnover. These results are in contrast with data reported by Hudson et al. (1995). For our studies, we used adult (240–250 g) Fisher 344 and Wistar rats (data not shown), and for the supranigral injections, we followed the coordinates and injection protocol described by Hudson et al. (1995). In addition to the lack of sprouting of TH-positive fibers in this study, we had reported earlier that repeated nigral GDNF injections did not affect the numbers of mesencephalic TH-immunopositive neurons and did not induce dopaminergic neuron sprouting in normal unlesioned animals (Beck et al., 1995). However, Hudson et al. (1995) did not provide any information about the actual weight or age of their experimental animals. A grossly different weight of the rats used in their studies compared with our animals would be likely to result in slightly different actual injection sites using identical stereotaxic coordinates. In addition, if the rats used by Hudson et al. (1995) were significantly younger, mesencephalic dopaminergic neurons could still be partially responsive to GDNF, or if they were much older, it might be possible that they had regained responsiveness. Recently, Tomac et al. (1995b) reported retrograde transport of iodinated GDNF from striatum to nigral dopaminergic cell bodies as well as GDNF immunoreactivity in dopaminergic neurons in adult rats. Although these results suggest a role for GDNF in adult rats, they do not provide direct evidence.

Our behavioral findings in early postnatal rats are very different from those produced by drugs inducing acute dopaminergic hyperfunction. Compounds such as amphetamine or cocaine, which induce the release of DA and 5-HT, produce a general locomotor hyperactivity of animals. Similar locomotor activation is observed after the injections of the DA precursor L-DOPA and of direct DA receptor agonists such as apomorphine (Greenberg et al., 1974; Beninger, 1983; Bergman et al., 1989). All these drugs result in the continuous stimulation of DA and 5-HT receptors, overriding the naturally occurring transmitter release. GDNF injections resulted in robust elevations of DA and 5-HT levels and of their metabolites in striatum and substantia nigra, in absence of evidence for morphological changes of nigrostriatal dopaminergic fibers and of serotonergic projections. The increase in

Table 5. Striatal [^{3}H]Mazindol Autoradiography in Rats with Intrastriatal GDNF Injections (5 μg, bilateral)

Striatal Level	Control (5)	Cytochrome C (5)	GDNF (5)
P8	0.84 ± 0.04	0.77 ± 0.07	1.08 ± 0.06[a]
P14	2.12 ± 0.19	2.22 ± 0.13	2.25 ± 0.20
P28	2.81 ± 0.25	2.92 ± 0.25	2.79 ± 0.22

Values are means ± SEM (in arbitrary OD units) obtained from sections at corresponding striatal levels. Slides were exposed to film, and OD was analyzed as described in Experimental Procedures. Numbers of rats analyzed are indicated in parentheses.
[a] Statistically significant difference compared with corresponding cytochome C injected group ($p < 0.01$; Student's t test).

mazindol binding at P8 could be due to a transient induction of DA uptake sites expression on dopaminergic terminals. Elevated transmitter levels in absence of sprouting indicate that existing terminals have a higher capacity to synthesize and release DA and 5-HT. We speculate that GDNF, rather than stimulating growth of dopaminergic neurites, increases the expression of specific genes of the transmitter machinery of dopaminergic and, as a secondary event, also of serotonergic neurons. The fact that GDNF administration elevated TH activity supports this view. Thus, it seems likely that GDNF injections as performed in the present study increase the capacity of dopaminergic and serotonergic neurons to release DA/5-HT in response to physiological stimuli by input neurons. The temporal pattern of stimulation of DA and 5-HT receptors in GDNF-treated animals is very different from that of animals treated with direct or indirect agonists, explaining the behavioral differences between the two types of treatments.

The slower weight gain in GDNF injected neonatal rats was most likely related to reduced milk intake based on the disadvantage of these rats in gaining and maintaining access to suckle. With the disappearance of the dystonia-like muscle spasms after 2 weeks, the GDNF injected rats quickly caught up with the control groups.

The specificity of GDNF remains to be established. Besides acting on dopaminergic and, indirectly, on serotonergic neurons, GDNF promotes survival and neurite growth of cultured peripheral neurons (Buj-Bello et al., 1995; Ebendal et al., 1995; Trupp et al., 1995) and increases the survival of chick and mammalian motor neurons in vitro and in vivo during development and following lesions (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995; Zurn et al, 1995). Results of studies using polymerase chain reaction analysis suggest that low levels of GDNF mRNA are expressed in many CNS areas, thus raising the possibility that GDNF might affect different CNS cells and multiple functions (Springer et al., 1994; Choi-Lundberg and Bohn, 1995; Ho et al., 1995). In particular for our study, it could be argued that the behavioral effects observed in the present study reflect direct GDNF actions on motor neurons. However, we can exclude this possibility based on our finding that unilateral striatal injections produced contralateral behavioral effects. Transport of GDNF from the striatal injection site to spinal cord motor neurons would have to occur via the cerebrospinal fluid through the ventricular system. It is impossible that side-specificity could be maintained during this process. There is further support for a direct action of GDNF on nigral dopaminergic neurons. Tomac et al. (1995b) demonstrated specific retrograde transport of GDNF in rats from striatum to substantia nigra. Additional data come from in vitro studies, where it has been established that, in mesencephalic cultures, GDNF acts on dopaminergic neurons without affecting serotonergic and GABAergic neurons (Lin et al., 1993; Poulsen et al., 1994). However, it remains to be established whether and which other neuron populations are directly responsive.

Taken together, our results suggest that, during postnatal development of the nigrostriatal dopaminergic projection, GDNF plays an important role in regulating dopamine synthesis, which in turn affects the synthesis of 5-HT, but GDNF does not act primarily as a tropic factor directing the developmental growth of dopaminergic fibers.

Experimental Procedures

GDNF Injections

Rat recombinant GDNF was produced, purified, and tested for biological activity as described previously (Beck et al., 1995). Neonatal rats (P2, Wistar) were cold anesthesized and put on a custom-made stage (modeling clay) that fixed the rats in a prone position. The stage was positioned in a stereotaxic frame. For injections, the skin over the skull was disinfected by wiping twice with 70% ethanol. Injections were done with Hamilton syringes into left and right striata at the following locations: 2.0 mm posterior from an imaginary line connecting the posterior end of left and right eye, 1.5 mm left and right from the midline, and 2.5 mm below the skin. At both sites, 5 μg of GDNF or control protein (cytochrome C) in 2 μl of each (10 μg in 4 μl total) of 0.9% saline with 10 mg/ml gentamicin at pH 7.4 were injected over 2 min. The location of the injections was verified in several control animals by injecting a 0.1% solution of trypan blue in 0.9% saline following this procedure. The trypan blue injected animals were sacrificed by decapitation while still anesthesized immediately after the injection, and their brains were removed. Most of the trypan blue was found in the parenchyma of both striata with traces in the cerebrospinal fluid (CSF) filling the lateral and third ventricles. The presence of traces of trypan blue in the CSF prompted us initially to use bilateral striatal injections and separate control animals instead of using unilateral admininstration with the contralateral side as control. In addition, a group of neonatal rats received unilateral striatal injections of GDNF or cytochrome C. In these animals, the injections were given in smaller portions over 4 min to avoid leakage of the injected solutions into the CSF.

Bilateral nigral injections were done with Hamilton syringes at the following coordinates: 6.5 mm posterior from an imaginary line connecting the posterior end of left and right eye, 1.0 mm left and right from the midline, and 2.5 mm below the skin. Injected volume, protein quantities, and duration of injection were identical to the ones described for striatal administration.

Adult rats (Fisher 344, 240–250 g) were given single unilateral injections of GDNF or control protein into the center of the striatum as described by Venero et al. (1994), and immediately dorsal to the zona compacta of the substantia nigra following the method described by Hudson et al. (1995).

Injected rats were taken for biochemical and morphological analysis at various intervals of time after the treatment. The anesthesized rats (50 mg/kg nembutal) were sacrificed by decapitation, and brains were removed onto ice. For biochemical and high pressure liquid chromatography analysis, left and right striatum and ventral mesencephalon (the area rostral of pons, caudal of the hypothalamus and anterior of the level of the aqueduct) were dissected. For [^{3}H]mazindol binding, brains were frozen in isopentane at −20°C. For histological analysis, anesthesized rats were perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde, and brains were removed, postfixed for 12 hr, and then sunk in 100 mM sodium phosphate (pH 7.4) with 25% sucrose and frozen.

Analysis of Dopamine, Serotonin, and Metabolites

Ventral mesencephalon and combined left and right striatum were frozen in 0.1 M perchloric acid on dry ice. Levels of endogenous dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), serotonin (5-HT), and hydroxyindolaceticacid (HIAA) in these tissues were determined by a simultaneous assay using high pressure liquid chromatography coupled to electrochemical detection using a modification of the procedure of Kilpatrick et al. (1986) as described previously by Irwin et al. (1990).

Enzyme Activities

Dissected brain areas were homogenized and then frozen in distilled water. Tyrosine hydroxylase (TH) and choline acetyltransferase (ChAT) activities and protein concentrations were as described by Knusel et al. (1990).

[^{3}H]GABA Uptake

The P2 synaptosome fraction of dissected and homogenized striatal tissue was prepared as described by Lapchak et al. (1993). Specific GABA uptake was determined using the protocol by Casper et al. (1991), as the fraction of uptake sensitive to 10 μM of the selective inhibitor NO-711 (95% of total uptake).

[^{3}H]Mazindol Binding to Brain Sections

Frontal sections (14 μm) of the anterior, medial, and posterior level of the striatum were cut in a cryostat and thaw-mounted on gelatin-coated microscopic slides. [^{3}H]Mazindol autoradiography was done according to the procedure described by Javitch et al. (1984) using 60 nM [^{3}H]mazindol (23.1 Ci/mmol; New England Nuclear–Dupont Research). Specific binding was defined by incubation in the presence of 10 μM nomifensine. Slides were exposed to X-ray film for 2 weeks. Autoradiographs were analyzed using an image analysis system: total striatal areas were outlined and specific optical densities (ODs) were determined by subtracting ODs measured on sections incubated with nomifensin from the OD determined in corresponding sections incubated with [^{3}H]mazindol alone.

Tyrosine Hydroxylase Immunohistochemistry

Frontal brain sections (45 μm) were cut on a sliding microtome and collected into PBS containing 0.02% NaN_3. Every tenth section spanning the striatum and every sixth section spanning the substantia nigra were stained for TH as described previously (Beck et al., 1995). To minimize section to section variations, during all steps of the immunohistochemistry, sections from one control, cytochrome C and GDNF injected rat were processed simultaneously, using staining racks that permitted incubation of all sections in the same solutions. Only sections that were stained together were used for histological comparisons.

Acknowledgments

Correspondence should be addressed to K. D. B. The study was supported by National Institutes of Health grants AG10480 (F. H.).

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Received August 14, 1995; revised December 28, 1995.

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